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(71) Applicant: MINNESOTA MINING AND MANUFACTURING COMPANY [US/US]; 3M Center, P.O. Box 33427, Saint Paul, MN 55133-3427 (US).

(72) Inventors: RASMUSSEN, Jerald, K.; P.O. Box 33427, Saint Paul, MN 55133-3427 (US). CURRY, Russell, A.; P.O. Box 33427, Saint Paul, MN 55133-3427 (US). WALKER, Margaret, M.; P.O. Box 33427, Saint Paul, MN 55133-3427 (US).

(74) Agents: BUSSE, Paul, W. et al.; Office of Intellectual Property Counsel, Minnesota Mining and Manufacturing Company, P.O. Box 33427, Saint Paul, MN 55133-3427 (US).

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(54) Title: HEPARIN FUNCTIONAL AFFINITY SUPPORTS

(57) Abstract

The disclosure describes hydrazide functional polymers obtained by reaction of azlactone functional polymers with hydrazine. The hydrazine functional polymers are useful to react with carbonyl functional materials such as heparin to provide heparin functional polymers which are useful for purification, preparation, separation and investigation of substances with biological activity. Methods of making and using the heparin functional polymers as significantly improved affinity chromatography supports such as beads for the purification of antithrombin III are described.

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HEPARIN FUNCTIONAL AFFINITY SUPPORTS

The present invention relates to polymers having hydrazide functional ligands on their surfaces and to heparin functional polymers prepared from such hydrazide functional polymers and methods of preparing and using both.

Background

Immobilized heparin is widely used as a ligand in affinity chromatography for purification, preparation, separation and investigation of substances with biological activity. Heparin is immobilized for chromatographic purposes by a variety of known processes onto solid supports. See, for example "Application of Immobilized Heparins for Isolation of Human Antithrombin III" by G. Mitra, E. Hall and I. Mitra, Biotechnology and Bioengineering, Vol. XXVIII, pp. 217-222 (1986).

East German Patent No. 276,814A1 describes coupling of nonderivatized heparin to hydrazide-derivatized polyacrylamide particles in an aqueous buffer at a pH less than or no greater than 3.8. This patent attributes improved coupling efficiency, greater stability of the coupling linkage and greater mechanical strength of the support to its process. However, the matrices claimed still have relatively poor mechanical stability and would be minimally useful for large scale separation processes where multiple uses and high flow rates are desired. In addition, the acidic conditions used for the preparation of the supports would result in some desulfation and hydrolysis of the heparin molecule, thus decreasing its biological specificity and usefulness for affinity chromatography purposes.

United States Patent No. 5,116,962 describes a process in which derivatized heparin is coupled to a carrier material containing reactive amino groups. However, this process requires the use of an alkali metal borohydride to provide the coupled product. Such borohydrides are expensive, toxic and hazardous and are considered environmentally unfriendly. Furthermore, the reaction times for the manufacture of the heparin functional support product are very long, preferably 12 to 16 days, resulting in a process with very low throughput.

Thus, it can be seen that recent efforts to provide improved heparin functional affinity chromatography supports are not very successful, and better supports and processes would be desirable.

The use of heparin functional affinity chromatography supports to purify antithrombin III is well known (see e.g. United States Patent No. 3,842,061), and it is the method of choice for providing antithrombin III for therapeutic use. Since antithrombin III is a very important but very expensive substance useful to treat cardiovascular disease, any significant improvement in processes for its isolation and purification is potentially very valuable. It is one of the objectives of the present invention to provide novel compositions and processes for the production of purified antithrombin III and other molecules with group specific affinity for heparin i.e., heparin-interactive molecules.

Summary of the Invention

Although the advantages of using immobilized heparin have been known for many years, there remain many disadvantages to the presently available methods and products which are reported or sold commercially.

A need exists for improved supports which provide:

a) High efficiencies of coupling heparin to a support;

b) High stability of the chemical linkage by which heparin is coupled;

c) Heparin ligands which have high biological activity;

d) Low nonspecific binding of molecules other than the target molecule to the heparin or the support matrix; and

e) High stability of the heparin functional affinity chromatography support to reagents used to remove nontarget proteins bonded to the heparin or the support.

The present invention provides improved heparin functional polymers and hydrazide functional polymers derived from azlactone functional polymers.

More particularly, the present invention provides improved heparin functional affinity chromatography supports. Such supports are based on hydrazide functional supports derived from azlactone functional supports. The supports of the invention provide a surprisingly high binding efficiency of target molecules to the heparin functional support, a stable coupling linkage,

mild reaction conditions to minimize degradation of heparin and high stability of the support to bases used to remove nontarget molecules from the heparin support.

This invention also provides hydrazide functional polymer supports derived from azlactone functional polymer supports.

Broadly, this invention also describes a method of providing a heparin functional surface comprising derivatizing an azlactone functional polymer surface by reacting with hydrazine, providing a hydrazide, and further derivatizing by reacting with heparin.

Broadly, the invention also describes a method of effecting interaction of a heparin-interactive molecule with a heparin functional surface comprising providing a surface which is an azlactone functional polymer which is derivatized by reaction with hydrazine to provide a hydrazide which is further derivatized by reaction with heparin and exposing the surface to the heparin-interactive molecule.

The term "surface" as used above is defined broadly as the outer or topmost boundary of a material.

The term "heparin" is used generally throughout this application and includes natural heparin, derivatized heparin, heparin salts, low molecular weight heparin, various chemically modified heparins which will react with hydrazide functional groups and synthetic heparin-like molecules. It will be understood by those skilled in the art that both carbonyl functional heparin and carbonyl functional heparin-like molecules will provide some or all of the advantages of the invention. The heparin used in most of the Examples of the invention is an aldehyde functional sodium heparin available commercially from Diosynth Laboratories Inc., (Chicago, IL). Other suitable commercially available heparins are available from Sigma Chemical Co., Calbiochem, and Scientific Protein Laboratories.

This invention provides improved processes for separating and purifying biologically active target molecules. More particularly, a surprisingly improved process for the purification of antithrombin III is described.

Detailed Description

European Patent Application No. 0392,735A2, based on U.S. Serial No. 07/335,835 which was filed in the United States on October 4, 1989 describes the use of azlactone-functional polymer supports useful for a variety of purposes including providing biologically active supports. Useful reactive polymers, e.g., polymeric supports, of the invention which are hydrazide functional may be prepared by reaction of known azlactone-functional polymers, e.g., polymer supports, such as those described in European Patent Application No. 0392,735A2, with hydrazine. The reaction is carried out under surprisingly mild conditions, i.e. at ambient temperature (about 25°C) and is rapidly completed (e.g. 1 to 24 hours, preferably 1 to 3 hours under laboratory conditions).

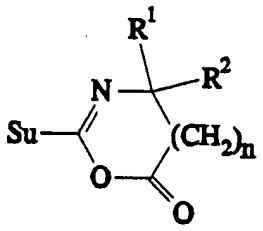
A preferred azlactone-functional polymer support for use in the present invention is commercially available as Emphaze® Biosupport Medium AB 1 from 3M, St. Paul, MN. The biosupport medium is prepared from commercially available methylene bis(acrylamide) monomer and 2-vinyl-4,4-dimethylazlactone as generally described in the European application cited above.

Generally the reaction is carried out in water (preferably) and/or a nonreactive solvent. An excess of hydrazine is conveniently used. Gentle warming can be used if it is desired to accelerate the reaction, but external warming is generally not needed. Although the reaction is generally carried out with azlactone functional beads, the azlactone functional polymer need not be in a bead form but can also be in any of the alternative forms such as films, matrices, membranes, coated membranes, other coated structures including organic and inorganic substrates and the like. Azlactone functional membranes and matrices are disclosed in United States Patent Application Serial Number 07/776,601 filed October 11, 1991, United States Patent Application Serial Number 07/896,107 filed June 9, 1992, and PCT Publication WO 93/06925. The azlactone-functional polymer supports used herein may also be prepared in situ and then reacted with hydrazine.

Although the hydrazide functional polymers are particularly useful for reaction of heparin as described herein, the hydrazide groups can also be advantageously reacted with other molecules containing aldehyde groups and with molecules containing ketone groups.

The hydrazide functional polymers of the invention can be made according to known procedures (see e.g. European Patent Application 0392,735A2 and United States Patent Applications 07/776,601 and 07/896,107) into supports and other useful forms e.g., beads, films, matrices, membranes, or coatings on organic or inorganic substrates.

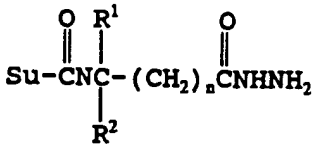
The preparation of hydrazide-functional reactive supports derived from azlactone-functional polymer supports can be carried out with reactive supports based on any of the known azlactone-functional supports which comprise an azlactone functional group of the Formula I



Formula I

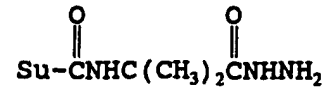
wherein R^1 and R^2 each independently are an alkyl group having 1 to 14 carbon atoms, a cycloalkyl group having 3 to 14 carbon atoms, an aryl group having 5 to 12 ring atoms, an arenyl group having 6 to 26 carbon and 0 to 3 S, N, and nonperoxidic O heteroatoms, or R^1 and R^2 taken together with the carbon to which they are joined form a carbocyclic ring containing 4 to 12 ring atoms, n is an integer 0 or 1 and Su represents the support.

Reaction of an azlactone functional support with hydrazine yields a hydrazide-functional support of the formula below:



where R^1, R^2, and n are described in Formula I above.

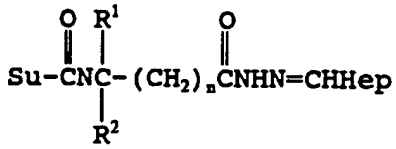
The preferred support group is obtained by reaction of an azlactone of Formula I in which R^1 and R^2 are methyl and n is zero with hydrazine to provide the hydrazide functional reactive support shown below.



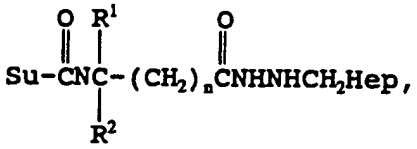
The reaction of the above described hydrazide-functional reactive support is carried out with heparin to bond the heparin covalently to the hydrazide group under very mild conditions, for example under ambient conditions (e.g. 25°C) in a buffered solution. Suitable buffers provide a pH range of about 5 to 10 and are e.g., sodium acetate, sodium phosphate and sodium carbonate in the presence or absence of salts, e.g. sodium sulfate. The reaction is relatively rapid and is usually essentially complete in less than 100 hours although small amounts of reaction continue for many hours e.g. up to 144 hours. Preferably the reaction time is 24 to 72 hours or less. The reaction may be accelerated by heating if desired.

The heparin used to provide the heparin functional affinity chromatography supports of the present invention contains reactive carbonyl groups such as aldehyde groups. Heparin preparations containing aldehyde groups are commercially available and certain of these may be used without further treatment to react with hydrazide functional reactive supports. Alternatively the number of carbonyl groups in commercially available naturally occurring heparin can be enhanced by simple oxidation of the heparin as described in the art, e.g. with a periodate salt. Some of the heparin preparations available commercially are chemically modified to enhance the number of aldehyde groups present.

Reaction of a hydrazide functional support with heparin yields a heparin-functional support of the formula below wherein Hep is a heparin residue.

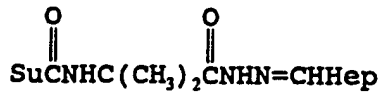


which can alternatively be reduced to

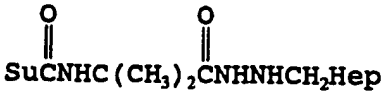


where R^1 and R^2 and n are described in Formula I above.

The preferred product is shown below:



An alternative product is shown below where the double bond at the heparin linkage is reduced by conventional methods such as reaction with sodium borohydride.



The heparin functional polymers prepared by the process of the present invention may be used in any convenient form such as films, membranes, coated membranes, beads or matrices or coated onto any desired support such as organic or inorganic supports, but are most conveniently used as beads in affinity chromatographic columns.

Such heparin functional beads, prepared as described above and in the Examples herein, are slurry packed into typical glass, metal or plastic chromatographic columns and equilibrated with buffer, e.g. commercially available 0.05 M tris(hydroxymethyl)aminomethane (TRIS) buffer, generally with added sodium chloride, e.g. at 0.1 to 0.5 M sodium chloride, preferably at about 0.15 M sodium chloride at or near neutral pH e.g. at pH 7.4. The column of beads is then subjected to a salt rinse by rinsing first with a volume of 0.05 M TRIS buffer containing 0.15 M sodium chloride (loading buffer), followed by an equal volume of 4.0 M sodium chloride in 0.05 M TRIS and finally using an equal volume of the loading buffer.

Purified antithrombin III (AT III), obtained from the American Red Cross, was used to quantitate the capacity of the columns to hold this substance. Depending upon the loading dose of heparin on the beads of the column, antithrombin III capacities as high as 11.10 mg/mL are observed when compared to commercial columns providing a capacity of 3.86 under the same conditions.

The use of the heparin functional affinity chromatography supports of the present invention is advantageously carried out on a large scale for the production of commercially useful amounts of AT III in large columns.

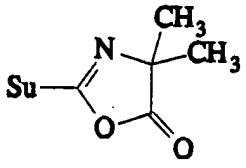
The heparin functional affinity chromatography columns of the invention have also been found to be useful in purification of human growth factors.

Heparin functional chromatographic supports are known to be gradually contaminated by molecules, e.g. proteins, other than the target molecules. Such nontarget molecules are said to undergo nonspecific binding which gradually blocks the heparin binding sites. The supports of the invention have been found to be significantly less susceptible to nonspecific binding than certain commercially available supports i.e. the supports of the invention have low nonspecific binding. Cleaning of nonspecific molecules from heparin supports is usually carried out with organic or inorganic bases. Inorganic bases are preferred because they are easier to remove from target molecules. The supports of the present invention have been found to demonstrate surprisingly good stability when washed with inorganic bases such as aqueous sodium hydroxide, retaining over 90% of their binding capacity when washed with 0.1 N aqueous sodium hydroxide and greater than 80% when washed with 1.0 N aqueous sodium hydroxide.

The following examples are used to illustrate the invention but are not intended to limit the invention as defined in the claims.

Example 1: Preparation of Hydrazide Functional Beads Derived From Azlactone Functional Groups on Methylene(bis)acrylamide Polymer

A 3.0 g portion of azlactone-functional beads having reactive units of the formula



wherein Su represents the polymeric support media (methylenebisacrylamide) which were prepared according to a variation (described hereinafter in Example 2) of the method of Example 4E of United States Application No. 335,835 and published as European Patent Application 0392,735A2 and

commercially available as Emphaze® Biosupport Medium AB 1 from 3M Company, St. Paul, MN, was weighed into a 50 mL centrifuge tube and 30 mL of 64% hydrazine in water was added. The mixture warmed slightly due to the initiation of reaction during addition and was stirred on a rotating mixer for two hours. The beads were then separated by filtration, washed repeatedly with distilled water until the wash water was neutral according to pH paper and stored in a refrigerator.

Example 2: Alternative Preparation of Hydrazide Functional Beads

An organic solution of 348 mL of heptane, 188 mL of toluene and 0.13 g of polymeric stabilizer as described in Example 4E of European Patent Application 0392,735A2 was heated with stirring to 35°C. To this stirred solution under a nitrogen atmosphere was added 0.72 mL of 2-vinyl-4,4-dimethylazlactone monomer. An aqueous solution was made from 13.33 g of methylenebis(acrylamide) available from Sigma Chemical Company, 90 mL of isopropyl alcohol, 60 mL of deionized water which was stirred at low heat until dissolved, then 0.55 g of sodium persulfate was added and the mixture was stirred until it dissolved.

The organic solution and the aqueous solution were mixed for 5 minutes, then 0.55 mL of tetramethylethylenediamine was added. Stirring was continued for two hours while azlactone functional beads formed. These beads could be isolated and used as synthetic intermediates, but in this run 20 g of hydrazine was then added to the reaction mixture and stirring was continued. After 1.5 hours the hydrazide functional beads were separated by filtration, washed thoroughly with distilled water until the wash water was neutral to pH paper and stored in a refrigerator.

Example 3: Preparation of Hydrazide Functional Beads-Hydrazine Concentration Study

Aliquots of Emphaze Biosupport Medium AB 1 beads (125 mg) were weighed into each of four separate Bio-Rad Poly-Prep® polypropylene columns and reacted with 5 mL of hydrazine solution in deionized water for two hours while being agitated on a rotating mixer. The breakoff tip on each column was removed, the excess hydrazine solution drained off, and the derivatized beads were washed with deionized water until the eluate was neutral to pH paper. The hydrazide content of the beads was measured according to the protocol described by G.T. Hermanson, A.K. Mallia, and P.K. Smith,

"Immobilized Affinity Ligand Techniques", Academic Press, San Diego, CA, 1992, pp. 287. The table lists the concentration of the derivatizing hydrazine solution and the corresponding measured hydrazide content of the derivatized beads.

Effect of Hydrazine Concentration on Bead Functionality

Table I

Hydrazine Concentration (moles/liter)	Hydrazide Content[1] (μmoles/mL)
0.2	18.4
0.5	19.7
1.0	20.0
3.0	21.3
Affi-Prep HZ[2]	1.8

[1] Average of two determinations
[2] Commercial Hydrazide functional bead from Bio-Rad Laboratories, Richmond, CA.

This example shows that very high hydrazide functionalities can be obtained from azlactone-functional beads even when using concentrations of hydrazine as low as 0.2 M for derivatization. By similar procedures, hydrazide functional supports can be prepared from other azlactone functional supports such as those described in Examples 5 of European Patent Application 0392,735A2.

Example 4: Preparation of Preferred Hydrazide Functional Beads

To a stirred solution of 32 mL of anhydrous hydrazine in 968 mL of distilled water was added slowly over 2.5 minutes 63 g of the lot of azlactone-functional beads used in Example 1. The mixture was stirred for 2 hours and 20 minutes, then the beads were separated by filtration using a sintered glass funnel and washed with distilled water until the water wash was neutral to pH paper. The beads were slurried with distilled water, allowed to settle for thirty minutes and the supernatant was poured off to remove unwanted extra fine beads. The procedure was repeated. A third repeat produced a clear

supernatant without fine beads. The measured hydrazide content was 22.4 micromoles per milliliter of beads. The desired hydrazide functional beads were stored in a refrigerator.

Example 5: Preparation of Heparin-Functional Beads

To 5 mL of buffered solution of 0.1 M aqueous sodium acetate (pH 5.0) and 5 mg/mL of sodium heparin (obtained from Diosynth, Chicago, IL as Batch No. 129) was added 1 mL of the hydrazide functional beads obtained in Example 1. After two hours of reaction the beads were isolated by filtration, washed with 5 mL of 0.1 M aqueous sodium acetate buffer, then washed with 10 mL of 1 M sodium chloride in 20 millimolar sodium phosphate (pH 7.0) then washed with distilled water.

To assess the presence of heparin adduct a sample of the beads was mixed with 0.5 mL of an aqueous toluidine blue solution (1% by weight). When the excess toluidine blue was removed by repeated washings with distilled water, the beads were reddish-purple in color, indicative of a characteristic heparin-toluidine blue reaction.

The reaction of heparin with hydrazide functional beads was repeated as described hereinabove except the proportion of reactants was 5 mL of buffered heparin and 2 mL of hydrazide functional beads. The heparin functional heads obtained were evaluated qualitatively and found to bind antithrombin III.

Example 6: Preparation of Heparin Functional Beads

Hydrazide functional beads prepared as described in Example 4 from commercially available Emphaze® Biosupport Medium AB 1 were reacted at three separate concentrations of heparin (25, 50 and 75 mg/mL) by adding the heparin solution in a volume equal to the volume of hydrazide-functional beads. The reaction was carried out by mixing at about 25°C in sodium acetate buffer, pH 5.0. After 72 hours the supernatant solution was discarded, the beads were rinsed with 10 volumes of water, followed by 10 volumes of 2.0 M sodium chloride at neutral pH, followed by 2 additional rinses with 10 volumes of water.

Example 7: Evaluation of Heparin Functional Beads for Antithrombin III Capacity

Separate columns were prepared to evaluate the beads prepared in Example 6 for antithrombin III capacity and to compare them to commercially available heparin-Sepharose® CL-6B beads (available from Pharmacia Biotech, Piscataway, NJ).

The heparin beads were slurry packed in glass columns (3 mm by 5 cm) and equilibrated and loaded using 0.15 M sodium chloride in 0.05 M TRIS buffer (available from Sigma Chemical Company, St. Louis, MO), pH 7.4. Each column of beads was subjected to rinsing in the FPLC® liquid chromatography instrument (available from Pharmacia) by rinsing with the loading buffer (3.5 mL), followed by 3.5 mL of 4.0 M sodium chloride in 0.05 M TRIS, pH 7.4 and reequilibrated with 3.5 mL of the loading buffer.

Purified antithrombin III from the American Red Cross was diluted to a concentration of 1.0 mg/mL in 0.15 M sodium chloride-0.05 M TRIS buffer. Twenty column volumes (7.0 mL) of antithrombin III were loaded onto each column on the FPLC system at a linear velocity of 100 to 200 cm per hour. In order to return to baseline absorbance, 3.5 mL (10 column volumes) of loading buffer were passed through the column. To elute the absorbed antithrombin III from the column a linear sodium chloride gradient was initiated and carried over 7.0 mL, starting with 0.15 M sodium chloride in TRIS buffer and ending with 2.0 M sodium chloride in TRIS buffer using calculated proportions of the loading buffer and the elution buffer (4.0 M sodium chloride in 0.05 M TRIS, pH 7.4). Fractions were collected and the antithrombin III capacity was quantitated from ultraviolet absorbance readings of the fractions at 280 nm. Bound antithrombin III began to elute at about 0.45 M sodium chloride, with peak elution at about 0.9 M.

The measured antithrombin III (ATIII) capacities were:

Table II

Coupling Dose of Heparin (mg/mL)	ATIII Capacity (mg/mL)
25	4.11
50	5.86
75	6.83
heparin-Sepharose® CL-6B (Pharmacia)	4.03

Example 8: Evaluation of Stability of Heparin Functional Beads to Washing with Various Concentrations of Aqueous Sodium Hydroxide

Heparin functional beads prepared according to the method of Example 6, which had been derivatized with 100 mg of heparin per mL solution, were used to prepare glass chromatographic columns (3 mm by 5 cm) after soaking the beads with mixing for two hours at 4°C in various concentrations of aqueous sodium hydroxide. The beads were then washed three times with water (5 mL water per 0.5 mL beads), then packed in columns to evaluate antithrombin III capacities. Purified antithrombin III was passed through each column as in Example 7 (using the FPLC® apparatus). A separately prepared heparin functional bead column which had been soaked in 20% ethanol in water to demonstrate stability of the column to a bacteriostatic medium was evaluated. A separately prepared heparin bead column which had been prepared using 0.15 M sodium chloride in 0.05 M TRIS buffer, pH 7.4 was used as the control.

The results are shown in Table III.

Table III

Type of Column	Treatment	ATIII Capacity as Percent of Control
Invention	0.1N NaOH	92
Invention	0.5N NaOH	90
Invention	1.0N NaOH	84
Invention	20% ethanol	103
Invention Control	Buffer	100

A second treatment was carried out by unpacking the beads from certain of the above columns, mixing in a treatment solution for two hours at 4°C, rinsing with water as described above, repacking the columns and again determining antithrombin III capacities. The results are shown in Table IV.

Table IV

Type of Column	Treatment	ATIII Capacity as Percent of Original Control
Invention	0.1N NaOH	92
Invention	20% ethanol	100

This experiment demonstrates excellent stability to sodium hydroxide washing of the invention beads.

Example 9: Evaluation of Additional Heparin Sources for the Preparation of Heparin-Functional Beads and Antithrombin III Capacity of the Beads

Heparin products of four descriptions (see Table V) were obtained from Scientific Protein Laboratories (Waunakee, WI). These heparin salts were dissolved at 100 mg/mL in 0.1 M acetate buffer, pH 5.0, and reacted with hydrazide functional beads from Example 4 at a 1:3 volume ratio of beads to heparin solution for 72 hours at about 25°C, with mixing. Rinsing of these heparin functional bead preparations was performed according to Example 6.

The heparin functional beads were packed into 3 mm by 5 cm chromatographic columns and tested for ATIII capacity, as described in Example 7. Resultant ATIII capacities were as follows:

Table V

Heparin Source	ATIII Capacity (mg/mL)
Heparin Sodium USP Lot No. 305G0910	11.10
Heparin Lithium USP Lot No. 301I0930	10.60
Heparin Sodium USP Lot No. 303I0910	9.69
Crude Sodium Heparin Lot No. MM0103893	5.28
heparin-Sepharose® CL-6B (Pharmacia)	3.86

Example 10: Evaluation of Nonspecific Binding on Heparin Functional Beads

A chromatographic column (3 mm x 5 cm) of heparin functional chromatographic support (heparin-Emphaze® support) beads (heparin at 100 mg/mL was reacted with hydrazide functional beads obtained as described in Example 6 at preferred conditions for reaction, reacted for 93 hours) was rinsed with loading buffer and elution buffer as in Example 7. Frozen human blood plasma (American Red Cross, Rockville, MD) was thawed at 4°C, centrifuged to remove cryo-precipitated material, and filtered through 0.8 μm and 0.2 μm seive sizes to remove additional particulates. 10.0 mL of plasma (29 column volumes) were loaded onto the chromatographic column at 200 cm/hr, followed by 10.0 mL of loading buffer to rinse the column. Non-specifically bound proteins were removed with 7.0 mL (20 column volumes) of 0.30 M sodium chloride in 0.05 M TRIS buffer, pH 7.4. Elution of ATIII was effected by a sodium chloride gradient step of 1.0 M sodium chloride with subsequent rinses of 2.0 M sodium chloride and 4.0 M sodium chloride to ensure complete removal of residual proteins. Fractions were collected during each step.

Absorbance readings of the fractions of the 0.30 M sodium chloride fraction are indicative of non-specifically bound proteins. In a side-by-side comparison with heparin-Sepharose® CL-6B, treated under the identical conditions, non-specifically bound proteins were reduced by about 35% on the heparin-Emphaze® support. This is qualitatively apparent in the chromatographic tracings, as the heparin-Emphaze® support returned more quickly to baseline absorbance after loading, and revealed a smaller peak of eluted proteins upon the 0.30 M sodium chloride rinse. Fractions eluted at 1.0 M sodium chloride showed a single band of protein consistent with ATIII using standard SDS-PAGE analysis with Coomassie Blue staining to identify the ATIII. No additional proteins were eluted with the 2.0 M or 4.0 M sodium chloride treatments.

Example 11: Time Course of Heparin Coupling Reaction

Hydrazide-activated Emphaze® Biosupport Medium AB 1 beads prepared according to Example 4 were reacted with heparin (available from Diosynth) at a challenge dose of either 5 mg/mL or 150 mg/mL according to the conditions in Example 5, (pH 5.0, 25°C, 0.1 M sodium acetate buffer). Beads were allowed to react with heparin for time increments as indicated in Table VI over 4 days, with beads being rinsed with water and high salt solution (2.0 M aqueous sodium chloride) at the end of each interval to stop the

coupling reacti n. The heparin-derivatized beads obtained were packed into chromatographic columns, and ATIII capacities were determined as in Example 7, using either 2 mg ATIII or 7 mg ATIII as the target molecule challenge.

The following results were obtained:

Table VI

Heparin Dose (mg/mL)	Time of Coupling (hours)	ATIII Capacity (mg/mL)
+ 5	0.25	<0.10
+ 5	1.2	0.17
+ 5	2	0.23
+ 5	12	0.85
+ 5	24	1.14
+ 5	48	1.27
+ 5	96	1.56
+ 150	0.25	0.95
+ 150	1.2	1.06
+ 150	2	1.42
+ 150	24	3.73
+ 150	48	3.95
+ 150	96	4.30
* 150	24	5.89
* 150	48	6.07
* 150	96	7.74

+ = 2 mg ATIII per column
* = 7 mg ATIII per column

Example 12: pH and Salt Effects on Heparin Coupling to Hydrazide Functional Beads

Heparin was coupled to hydrazide-activated Emphaze® Biosupport Medium AB 1 beads prepared according to Example 4 at three pH levels and in increasing molarities of aqueous sodium sulfate, using a heparin challenge of 100 mg/mL and a 48-hour coupling time. The buffers used were dependent on the desired pH, with coupling buffer of 0.1 M sodium acetate at pH 5.07, 0.1 M sodium phosphate at pH 7.0, and 0.1 M sodium carbonate at pH 10.0.

The following results were obtained:

Table VII

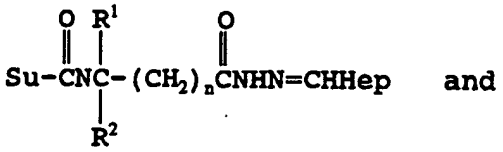
pH	Concentration of Salt (Sodium Sulfate)	ATIII Capacity (mg/mL)
5.07	0	7.84
5.07	0.125 M	8.12
5.07	0.25	6.72
5.07	0.50	6.48
5.07	1.00	6.09
7.00	0	5.59
7.00	0.125 M	6.28
7.00	0.25	6.26
7.00	0.50	6.37
7.00	1.00	6.69
10.00	0	2.23
10.00	0.125 M	1.88
10.00	0.25	2.13
10.00	0.50	2.58
10.00	1.00	2.71

The experiment demonstrates that the heparin coupling reaction was more efficient in terms of producing high ATIII capacity at about pH 5 or about pH 7 than at about pH 10. The salt effect varied with pH.

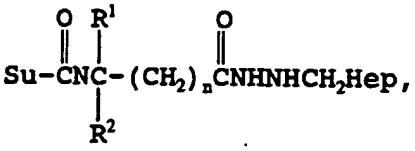
Claims

1. A heparin functional polymer comprising biologically active heparin covalently bonded to a hydrazide reactive group formed from reaction of hydrazine with an azlactone-functional polymer.

2. A heparin functional polymer according to claim 1 of the formula selected from



and

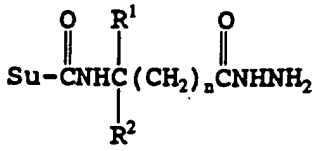


where Su is a support, n is zero or one, Hep is a heparin residue and R^1 and R^2 each independently is an alkyl group having 1 to 14 carbon atoms, a cycloalkyl group having 3 to 14 carbon atoms, an aryl group having 5 to 12 ring atoms, an arenyl group having 6 to 26 carbon and 0 to 3 S, N, and nonperoxidic O heteroatoms, or R1 and R2 taken together with the carbon to which they are joined can form a carbocyclic ring containing 4 to 12 ring atoms.

3. The heparin function polymer according to claim 1 wherein the heparin bonded to the support retains more than 80% antithrombin-III binding activity after washing the support with 1 N sodium hydroxide.

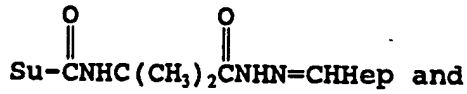
4. The heparin function polymer according to claim 1 wherein the polymer binds more than about 5 mg/mL antithrombin-III.

5. A hydrazide functional chromatographic support comprising

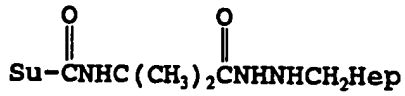


where n is zero or one and where R1 and R2 each independently is an alkyl group having 1 to 14 carbon atoms, a cycloalkyl group having 3 to 14 carbon atoms, an aryl group having 5 to 12 ring atoms, an arenyl group having 6 to 26 carbon and 0 to 3 S, N, and nonperoxidic O heteroatoms, or R1 and R2 taken together with the carbon to which they are joined can form a carbocyclic ring containing 4 to 12 ring atoms, and Su is a support.

6. The heparin functional chromatographic support according to claim 5 selected from the group consisting of



and



wherein Su is a support and Hep is a heparin residue.

7. A method of effecting interaction of a heparin-interactive molecule with a heparin functional surface comprising the steps of 1) providing a surface comprising azlactone functional polymer which is derivatized by reaction with hydrazine to provide a hydrazide which is further derivatized by reaction with heparin and ii) exposing the surface to the heparin-interactive molecule.

8. A method according to claim 7 wherein the heparin-interactive molecule is antithrombin III or a human growth factor.

INTERNATIONAL SEARCH REPORT

Inter. .onal Application No
PCT/US 94/09149

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C08B37/10 B01J20/26 C12N11/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C08B B01J C12N A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	EP,A,0 295 073 (CHROMATOCHEM INC) 14 December 1988 see page 6; tables 1,No,9 see page 12; example 10; table 2	1-8
Y	DD,A,276 814 (FORSCHUNGSZENTRUM FÜR TIERPRODUKTION) 14 March 1990 cited in the application	1-8
A	EP,A,0 203 463 (BEHRINGWERKE) 3 December 1986	

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
24 November 1994	15. 12. 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Lensen, H

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PATENT ABSTRACTS OF JAPAN vol. 16, no. 261 (C-950) 12 June 1992 & JP,A,04 059 796 (CHISSO CORP) 26 February 1992 see abstract & DATABASE WPI Week 9215, Derwent Publications Ltd., London, GB; AN 118378 see abstract	
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